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A Polyketide Synthase Gene Required for Biosynthesis of Fumonisin Mycotoxins in *Gibberella fujikuroi* Mating Population A

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Robert H. Proctor, Anne E. Desjardins, Ronald D. Plattner, and Thomas M. Hohn. 1999. A Polyketide Synthase Gene Required for Biosynthesis of Fumonisin Mycotoxins in *Gibberella fujikuroi* Mating Population A. *Fungal Genetics and Biology* 27, 100–112. Fumonisins are toxins associated with several mycotoxicoses and are produced by the maize pathogen *Gibberella fujikuroi* mating population A (MP-A). Biochemical analyses indicate that fumonisins are a product of either polyketide or fatty acid biosynthesis. To isolate a putative polyketide synthase (PKS) gene involved in fumonisin biosynthesis, we employed PCR with degenerate PKS primers and a cDNA template prepared from a fumonisin-producing culture of *G. fujikuroi.* Sequence analysis of the single PCR product and its flanking DNA revealed a gene (*FUM5*) with a 7.8-kb coding region. The predicted *FUM5* translation product was highly similar to bacterial and fungal Type I PKSs. Transformation of a cosmid clone carrying *FUM5* into *G. fujikuroi* enhanced production in three strains and restored wild-type production in a fumonisin nonproducing mutant. Disruption of *FUM5* reduced fumonisin production by over 99% in *G. fujikuroi* MP-A. Together, these results indicate that *FUM5* is a PKS gene required for fumonisin biosynthesis.

Index Descriptors: Gibberella fujikuroi; Fusarium moniliforme; polyketide synthase; toxin; mycotoxin biosynthesis.

Fumonisins are mycotoxins that frequently contaminate maize and are associated with a number of mycotoxicoses, including leucoencephalomalacia in horses, pulmonary edema in swine, and liver cancer in rats (Nelson *et al.*, 1993). Human esophageal cancer has also been epidemiologically correlated with consumption of fumonisin-contaminated maize in certain areas of South Africa and China (Marasas, 1996). Fumonisins are produced by a number of species within the *Gibberella fujikuroi* species complex (Desjardins and Proctor, 1998). Production by *G. fujikuroi* mating population A (MP-A) (anamorph *Fusarium moniliforme*, syn. *F. verticillioides*) is of particular concern because of the prevalence of this fungal species on maize worldwide (Munkvold and Desjardins, 1997). *G. fujikuroi* MP-A is one of the most common pathogens of maize and is associated with diseases of the roots, stalk, and ears. In addition, the fungus is frequently present in apparently healthy maize tissues (Munkvold and Desjardins, 1997).

Structurally, fumonisins consist of a linear 19- or 20-carbon backbone with hydroxyl, methyl, and tricarballylic acid moieties at various positions along the backbone (Fig. 1). In the B series of fumonisins there is an amino group at

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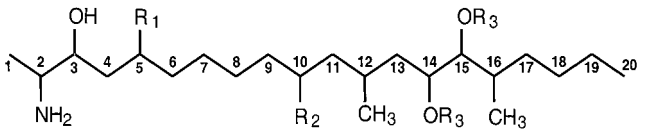

	R_1	R_2
Fumonisin B_1	OH	OH
Fumonisin B_2	OH	H
Fumonisin B_3	H	OH

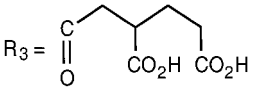

FIG. 1. Structure of B series of fumonisins.

carbon atom 2 (C-2), while in other series the amino group can be acetylated or substituted with hydroxypyridine (Bezuidenhout *et al.*, 1988; Musser *et al.*, 1996; Musser and Plattner, 1997). The B series of fumonisins are typically the most abundant fumonisins that occur in naturally infected maize kernels, with fumonisin B_1 (FB_1) usually making up approximately 70% and fumonisins B_2 (FB_2) and B_3 (FB_3) each making up 10–20% of the total fumonisin content (Nelson *et al.*, 1993). Feeding studies with a variety of precursors indicate that different components of the FB_1 molecule have diverse biogenic origins. Specifically, the oxygen atoms at C-3, C-5, C-10, C-14, and C-15 are derived from molecular oxygen (Caldas *et al.*, 1998), the tricarballylic moieties at C-14 and C-15 may be derived from glutamic acid via the citric acid cycle (Blackwell *et al.*, 1996), the methyl groups at C-12 and C-16 are derived from methionine (Plattner and Shackelford, 1992), the amino group and C-1 and C-2 of the fumonisin backbone are derived from alanine (Branham and Plattner, 1993), and C-3 to C-20 of the backbone are derived from acetate (Blackwell *et al.*, 1994).

The FB_1 backbone is structurally very similar to sphinganine, a fatty acid-derived intermediate in sphingolipid metabolism (Merrill *et al.*, 1997). However, it is not clear whether fumonisins are derived from a fatty acid or polyketide because both types of compounds are derived from acetate. Fatty acid biosynthesis typically begins with the condensation of acetyl and malonyl, which is derived from acetate, and the concomitant release of carbon dioxide (Wakil, 1989). The product of this reaction is a linear, 4-carbon molecule with carbonyl groups at C-1 (α) and C-3 (β). The β-carbonyl is processed, first by reduction to a hydroxyl, then dehydration to an enoyl function, and finally reduction to an alkyl function (Wakil, 1989). The resulting 4-carbon product, with the C-1 carbonyl intact, undergoes condensation with another malonyl followed by processing of the β-carbonyl to an alkyl function. This cycle of condensation with malonyl followed by carbonyl processing is repeated a precise number of times until a full-length carbon chain is formed. Polyketide biosynthesis occurs via the same mechanism except β-carbonyl groups are often left unprocessed or only partially processed. In addition, polyketide biosynthesis sometimes utilizes substrates other than acetyl and malonyl and generally involves further processing (e.g., cyclization or addition of side groups) after the carbon chain is formed (Hopwood and Sherman, 1990; Katz and Donadio, 1993).

Type I fatty acid synthases (FASs) and polyketide synthases (PKSs) are multifunctional peptides that include all the functional domains necessary to complete the formation of full-length fatty acids or polyketides (Hopwood and Sherman, 1990; Wakil, 1989). The β-ketoacyl synthase (KS), acyl transferase (AT), and acyl carrier protein (AC) domains participate in the condensation reactions, while the β-ketoacyl reductase (KR), dehydratase (DH), and enoyl reductase (ER) domains participate in β-carbonyl processing. Animal FASs and some fungal and bacterial Type I PKSs also have a thioesterase (TE) domain and fungal FASs have a malonyl/palmityl transferase (M/PT) domain. These TE and M/PT domains participate in the release of the full-length fatty acid or polyketide from the enzyme. Fungal FASs consist of two peptide subunits encoded by different genes. The KS, KR, and AC domains are located on the α-subunit, while the AT, ER, DH, and M/PT domains are present on the β-subunit (Hopwood and Sherman, 1990). In contrast, fungal PKSs consist of a single peptide with KS, AT, and AC domains and sometimes one or more of the DH, ER, KR, and TE domains. The presence of the DH, ER, and KR domains depends on whether and the extent to which β-carbonyls are processed during polyketide biosynthesis.

Virtually nothing is known about the molecular genetics of fumonisin production. Classical genetic studies with natural variants and laboratory mutants of *G. fujikuroi* MP-A have identified four linked loci, *fum1*, *fum2*, *fum3*, and *fum4*, that are involved in fumonisin biosynthesis (Desjardins *et al.*, 1996, 1992; Plattner *et al.*, 1996). Strains defective at the *fum1* locus do not produce fumonisins (Desjardins *et al.*, 1992) while strains defective at *fum2* lack the ability to hydroxylate C-10 of the fumonisin backbone and, therefore, produce FB_2 but not FB_1 or FB_3 (Desjardins *et al.*, 1996). Similarly, strains defective at *fum3* lack the ability to hydroxylate C-5 and, therefore, produce FB_3 but not FB_1 or FB_2 (Desjardins *et al.*, 1996).

In addition, strains defective at *fum4* produce only low levels of FB_1, FB_2, and FB_3 (Plattner *et al.*, 1996).

The current understanding of the biochemistry and molecular genetics of fatty acid and polyketide biosynthesis has the potential to provide insight into fumonisin biosynthesis, including approaches to isolate the gene(s) involved in the formation of the fumonisin backbone. Comparisons of the predicted peptide sequences of PKSs from fungal and bacterial sources have revealed regions of conserved amino acids within the various functional domains (Keller *et al.*, 1995). Such amino acid conservation has been exploited in attempts to isolate fungal PKS genes (Keller *et al.*, 1995; Feng and Leonard, 1995). To explore the possibility that the fumonisin backbone is a polyketide rather than a fatty acid, we employed a PCR approach that included a cDNA template and degenerate primers with sequences based on the KS domains of fungal and bacterial Type I PKSs. The approach yielded a PCR product that was part of a PKS gene required for fumonisin biosynthesis.

MATERIALS AND METHODS

Strains and media. The wild-type and fumonisin production mutants used in this study are listed in Table 1. Media employed in this study were GYAM, GYP (2% glucose, 1% peptone, and 0.3% yeast extract) (Hohn and Desjardins, 1992), V-8 juice agar (Tuite, 1969), and cracked maize kernels (Leslie *et al.*, 1992). The latter medium was prepared by combining 10 g cracked maize kernels and 2.5 ml distilled water in a 50-ml Erlenmeyer flask, autoclaving for 20 min, and then adding an additional 2.0 ml sterile water. GYAM medium was a modification (Plattner and Shackelford, 1992) of the medium described by (Clouse *et al.*, 1985) and contained 0.24 M glucose, 0.05% yeast extract, 8.0 mM L-asparagine, 5.0 mM malic acid, 1.7 mM NaCl, 4.4 mM K_2HPO_4, 2.0 mM $MgSO_4$, and 8.8 mM $CaCl_2$. To prepare this medium, the salts and yeast extract were combined with water at 2× concentration, adjusted to pH 3 with phosphoric acid, and autoclaved. This solution was combined with 0.4 vol of a 1.2 M glucose solution (autoclaved separately), 0.2 vol of a 50 mM malic acid solution (filter sterilized), and 0.4 vol of sterile water (Plattner and Shackelford, 1992).

TABLE 1
Gibberella fujikuroi Strains

Strain No.	Fumonisin production	Genotype	Reference
M-3120	FB_1, FB_2, FB_3	*fum1, fum2, fum3*	(Leslie *et al.*, 1992)
M-3125	FB_1, FB_2, FB_3	*fum1, fum2, fum3*	(Leslie *et al.*, 1992)
109-R-20	FB_2	*fum1, fum2*$^-$, *fum3*	(Desjardins *et al.*, 1996)
575-R-5	FB_3	*fum1, fum2, fum3*$^-$	Proctor, Desjardins, and Plattner, unpublished
57-7-7	None	*fum1*$^-$, *fum2, fum3*	(Desjardins *et al.*, 1996)

Fumonisin assays. Fumonisin production was assessed in cracked maize kernel and GYAM cultures. GYAM is a semidefined medium but supports only low levels of fumonisin production. Cracked maize consistently supports high levels of production, but is undefined and occasionally contains low background levels of fumonisins. Both media were inoculated with a single mycelial plug taken from V-8 juice agar cultures. GYAM cultures were grown in Erlenmeyer flasks shaken 2 weeks at 200 rpm in the dark at 28°C. Cultures were filtered through 0.2-μm cellulose acetate membranes (Nalgene) prior to analysis. Cracked maize cultures were grown in the dark at 28°C and extracted with 25 ml acetonitrile:water (1:1, v:v) as previously described (Desjardins *et al.*, 1994). GYAM culture filtrates and cracked maize culture extracts were analyzed for fumonisins by either HPLC or HPLC- mass spectrometry as previously described (Desjardins *et al.*, 1994; Plattner *et al.*, 1996).

Nucleic acid manipulations. Genomic DNA was isolated from *G. fujikuroi* via a previously described miniprep protocol (Desjardins *et al.*, 1996). RNA was isolated from liquid GYAM cultures by filtering the cultures through Whatman No. 4 filter paper and immediately grinding approximately 1 g of the mycelial mat in liquid nitrogen. The ground mycelium was added to 10 ml of TRIZOL Reagent (GIBCO BRL), vortexed, combined with 2 ml chloroform, vortexed again, incubated for 2 min at room temperature, and then centrifuged at 4°C for 15 min at 12,000*g*. RNA was precipitated from the resulting aqueous phase by adding 5 ml isopropyl alcohol, mixing, incubating 10 min, and centrifuging at 4°C for 10 min at 12,000*g*. The pellet was washed with 75% ethyl alcohol and resuspended in 200 μl diethyl pyrocarbanate-treated water. Messenger RNA was purified using the Micro-Poly(A) Pure protocol (Ambion) and cDNA was prepared as described in the Advantage cDNA PCR kit (Clontech).

PCR was carried out in a Perkin–Elmer 2400 thermocycler using either *Pfu* (Stratagene) or *Taq* DNA polymer-

ases. PCR products were cloned into pCR2.1 (Invitrogen) as specified by the manufacturer. D. Brown and N. Keller (Texas A&M University) kindly provided nucleotide sequences for the degenerate β-ketoacyl synthase domain primers, KS1 and KS2 (Keller *et al.*, 1995). The sequence of primer KS1 was 5′-GGRTCNCCIARYTGIGTIC-CIGTICCRTGIGC-3′ and the sequence of primer KS2 was 5′-MGIGARGCIYTIGCIATGGAYCCICARCARMG-3′, where I indicates inosine, M indicates A or C, R indicates A or G, Y indicates C or T, and N indicates A, C, G, or T. PCR conditions used with these primers were 94°C denaturation for 20 s (first cycle 1 min), 53°C annealing for 20 s, and 72°C extension for 40 s (last cycle 4 min) for a total of 30 cycles.

Nucleotide sequences of both DNA strands were determined via the ABI PRISM Dye Terminator Cycle sequencing Kit (Perkin–Elmer). Sequencing templates consisted of Midiprep (Qiagen) purified plasmids or agarose gel/Ultra Clean (Mo Bio) purified PCR products. Primers for sequencing included the M13 forward and reverse primers as well as primers specific for *G. fujikuroi* DNA. DNA probes for hybridization were radiolabeled with Prime-a-Gene (Promega).

Cosmid library preparation. A *G. fujikuroi* MP-A cosmid library was prepared from cosmid vector pSuperCos P1 (Hohn *et al.*, 1993) and genomic DNA of strain M-3125. The vector consists of pSuperCos (Stratagene) carrying the chimeric hygromycin resistance gene (*Hyg*) constructed from *Cochliobolus heterostrophus* promoter 1 and the coding region of the *Escherichia coli* hygromycin phosphotransferase gene (Turgeon *et al.*, 1987). Genomic DNA was prepared by filtering GYP-grown cultures of M-3125 through Whatman No. 1 filter paper, grinding 5 g of the resulting mycelial mat in liquid nitrogen, and resuspending in 8 ml extraction buffer (200 mM Tris, pH 8.5, 250 mM NaCl, 25 mM EDTA, pH 8.0, and 0.5% SDS) (Hohn and Desjardins, 1992). The resulting suspension was extracted twice with an equal volume of phenol: chloroform (1:1, v:v) and twice with chloroform. The aqueous phase was incubated first with RNase for 90 min at 37°C, then on ice for 15 min, and then combined with an equal volume of 5 M lithium chloride. After an additional 20-min incubation on ice, the solution was centrifuged at 22,000*g* for 20 min. The supernatant was combined with 2.5 vol cold ethyl alcohol. The precipitated DNA was removed from the aqueous solution without centrifugation, rinsed with 70% ethyl alcohol, and resuspended in Tris–EDTA buffer (Sambrook *et al.*, 1989). The DNA preparation was digested with restriction enzyme *Mbo*I and ligated into pSuperCosP1 following the pSuperCos protocol and packaged in the Gigpack II XL Packaging Extract (Stratagene) as specified by the manufacturer.

Disruption vector construction. Construction of the disruption vector began with a 4.7-kb *Bam*HI fragment from cosmid clone 16-1 cloned into the *Bam*HI site of pBluescriptII KS. Partial sequence analysis indicated that this fragment was from within the putative PKS gene coding region. This plasmid (pB6) was digested with *Cla*I/*Sma*I to excise the *Hind*III site within the pBluescriptII KS polylinker region. The digested pB6 was purified via agarose gel electrophoresis and QiaQuick (Qiagen) and religated to yield plasmid pB6CS. A 530-bp fragment from near the middle of the pB6CS insert was excised via *Hind*III digestion followed by agarose gel electrophoresis and QiaQuick purification. The resulting 7.2-kb product was then ligated to a 2.5 kb *Hind*III fragment carrying the *Hyg* marker gene (Turgeon *et al.*, 1987) from pUCH4 (Hohn, unpublished). The resulting plasmid was designated pB6CS-Hyg.

Transformation. The protocol used to isolate and transform protoplasts was similar to that described by Salch and Beremand, (1993) with the following modifications. Conidia were germinated only until germ tubes were two to five times the length of microconidia (typically for 8–12 h at 28°C). Cell wall digestions were carried out at 30°C in 20 ml of 0.7 M NaCl containing 5 mg/ml Novozyme (Sigma), 25 mg/ml driselase, and 0.5 mg/ml chitinase. After two washes with STC buffer (Salch and Beremand, 1993), protoplasts were diluted to 1×10^8 protoplasts per ml in STC:SPTC:DMSO (8:2:0.1, v:v:v) and frozen at −80°C as described by Royer *et al.* (1995). For transformation, 1–5 μg of cosmid or plasmid DNA was used per 10^7 protoplasts. The protoplast–DNA solution was mixed with 4.5 ml molten regeneration medium and dispensed over the surface of 20 ml solidified regeneration medium. After an overnight incubation, this regeneration medium was overlaid with 10 ml of 1% water agar amended with 150 μg hygromycin B/ml. The final concentration of hygromycin in the 35 ml of selection medium (regeneration medium and overlay) in each plate was 45 μg/ml.

RESULTS

Amplification of β-ketoacyl synthase DNA fragment. Our PCR strategy to isolate a putative PKS gene involved in fumonisin biosynthesis employed degenerate

primers and a cDNA template. The sequences of the degenerate primers, KS1 and KS2, were based on two regions of conserved amino acids in the β-ketyoacyl synthase domains of Type I fungal and bacterial PKSs (Keller *et al.*, 1995). The cDNA templates were prepared from 100 ml GYAM cultures of *G. fujikuroi* MP-A strain M-3125. We postulated that the transcript for a PKS involved in fumonisin biosynthesis would be relatively abundant during the time when fumonisins are first detected in liquid culture. Preliminary experiments indicated that there is considerable variation in timing of the initiation of fumonisin production in GYAM cultures of strain M-3125. The time at which fumonisins were first detected in the cultures varied between experiments and between cultures within experiments and ranged from 54 and 92 h after inoculation. Because of this variation, we isolated RNA from GYAM cultures at 42, 52, 64, 76, 88, and 100 h after inoculation. Culture filtrates from these time points were also analyzed for their fumonisin content via HPLC. Fumonisins were detected at 76, 88, and 100 h (Fig. 2). cDNA was prepared from the 64 and 88 h RNA samples to provide cDNA templates from a culture in which fumonisin production was occurring (88 h) and from a culture in which fumonisin production had not yet begun (64 h).

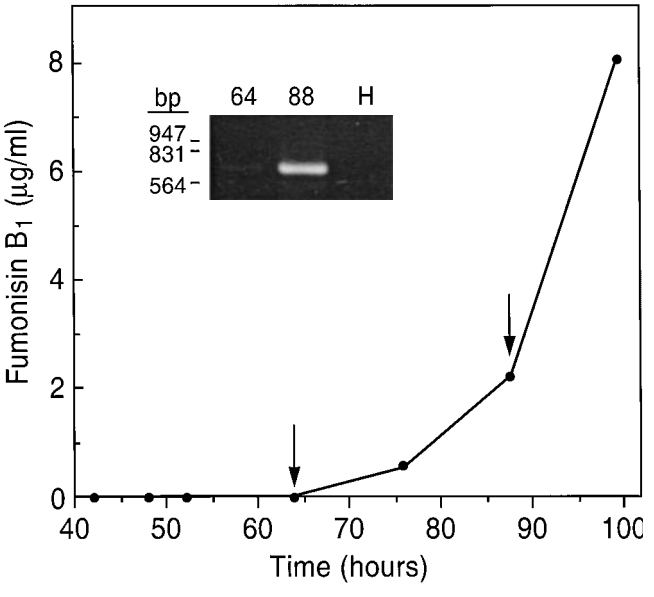

FIG. 2. FB_1 production in GYAM culture of *G. fujikuroi* strain M-3125. Arrows indicate time points from which cDNA was prepared. Inset, agarose gel electrophoresis of amplification products from with KS1/KS2 primers and cDNA templates. Lanes: 64, 64 h cDNA template; 88, 88 h cDNA template; H, water control.

In PCR experiments employing KS1 and KS2 as primers and the 64- and 88-h cDNA preparations as templates, a ~650-bp product was amplified (Fig. 2) and designated FMKS. This amplification product was at least sixfold more abundant when amplified from the 88-h cDNA template than from the 64-h template. The sequences of three independent FMKS clones were identical and BLAST analysis of the predicted amino acid sequence revealed a high degree of similarity to KS domains from bacterial and fungal Type I PKSs and animal FASs.

Identification and analysis of polyketide synthase gene. Four cosmid clones carrying FMKS were isolated via standard protocols (Sambrook *et al.*, 1989). Nucleotide sequence analysis of five overlapping subclones derived from cosmid clones 2-2 and 16-1 revealed the presence of a 7.82-kb open reading frame (ORF) interrupted by five introns ranging in size from 57 to 85 bp. BLAST analysis (Altschul *et al.*, 1990), using the National Center for Biotechnology Information (NCBI) nonredundant protein database, revealed that the predicted 2607-amino-acid translation product (Fig. 3) had a high degree of similarity to numerous Type I PKSs and several animal FASs. The highest level of similarity (BLAST Score 597, $P = 10^{-254}$) was with *PKS1* from the filamentous fungus *C. heterostrophus* (Yang *et al.*, 1996). Highly significant ($P = 10^{-103}$–10^{-137}) levels of similarity to prokaryotic PKSs from organisms such as *Mycobacterium tuberculosis, M. leprae, Streptomyces hygroscopicus,* and *Saccharopolyspora erythrae* were also evident, while lower, but still significant, levels of similarity ($P = 10^{-97} - 10^{-51}$) were observed with other fungal PKSs in the NCBI database. Analysis of the predicted translation product of the *G. fujikuroi* PKS gene also revealed the presence of six functional domains characteristic of Type I PKSs (Yang *et al.*, 1996). These domains were (from N terminus to C terminus) KS, AT, DH, ER, KR, and AC (Fig. 3 and 4). There was no region with a conserved amino acid sequence indicative of a TE domain.

The presence of five introns was initially suspected via the presence of nucleotide sequences that closely matched the 5′ and 3′ end splice and internal consensus sequences for fungal introns (Gurr *et al.*, 1987). Splicing the putative introns from the sequence eliminated stop codons that otherwise interrupted the ORF. The presence of the introns was confirmed by PCR amplification of DNA fragments that spanned the intron positions from cDNA and genomic DNA templates. As expected, the PCR products amplified from the cDNA template were smaller, by the appropriate amount, than the products amplified

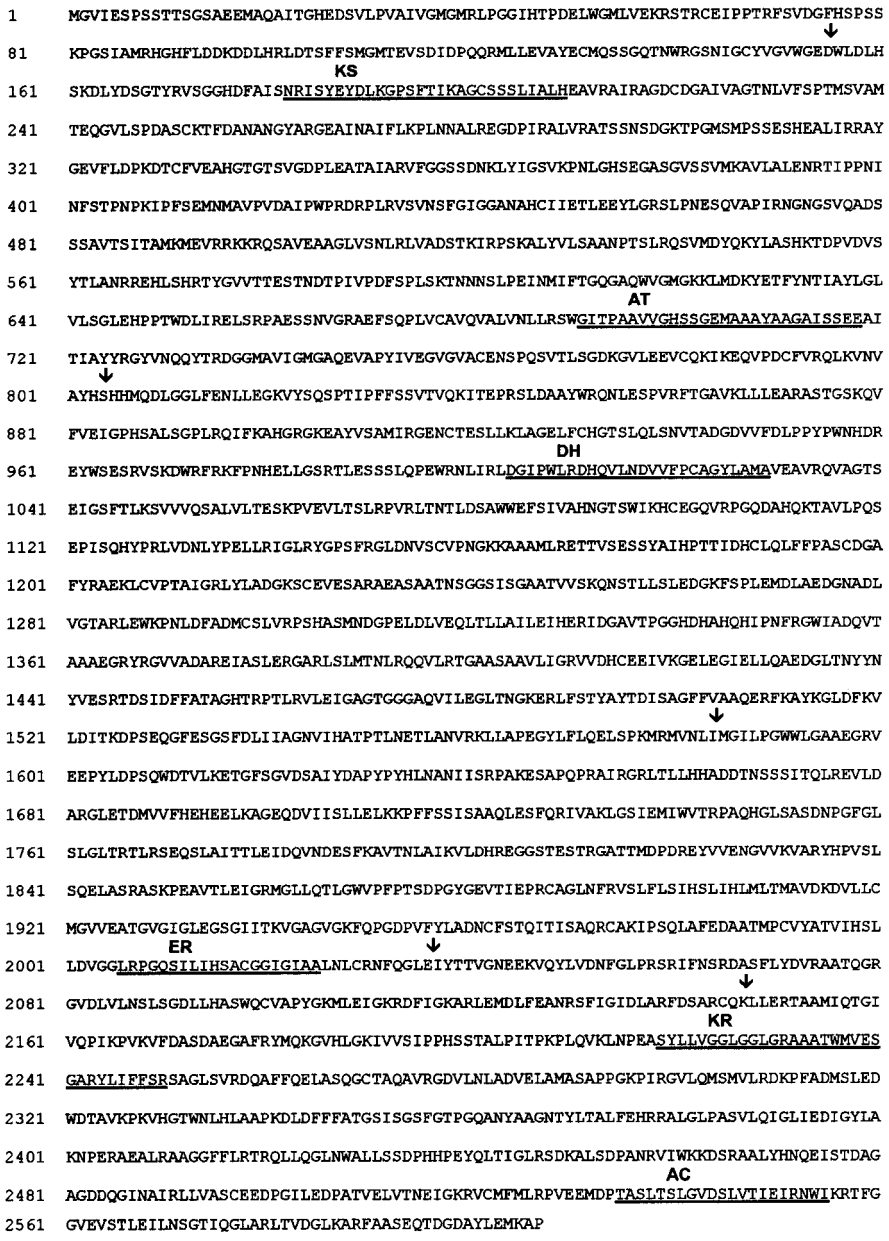

FIG. 3. Predicted amino acid sequence of the *G. fujikuroi FUM5* translation product showing regions corresponding to six putative functional domains (underlined). Arrows indicate intron positions in the corresponding nucleotide sequence of *FUM5.* The GenBank accession number for *FUMS* is AF155773.

from the genomic DNA template. Nucleotide sequence analysis also confirmed the absence of the intron sequences in the cDNA amplification products.

In keeping with the proposed genetic nomenclature for plant pathogenic fungi (Yoder *et al.*, 1986), the *G. fujikuroi* MP-A PKS gene has been designated *FUM5.* However, if further information indicates that this gene is equivalent to a previously identified *fum* locus (see below) it may be necessary to alter the *FUM5* designation.

Complementation of a fumonisin production mutant. Restriction enzyme analyses indicated that cosmid clone 16-1 had the entire *FUM5* coding region and 17 kb of 5′ flanking DNA and 2 kb of 3′ flanking DNA. This analysis also indicated that cosmid clone 2-2 consisted of 6.5 kb of

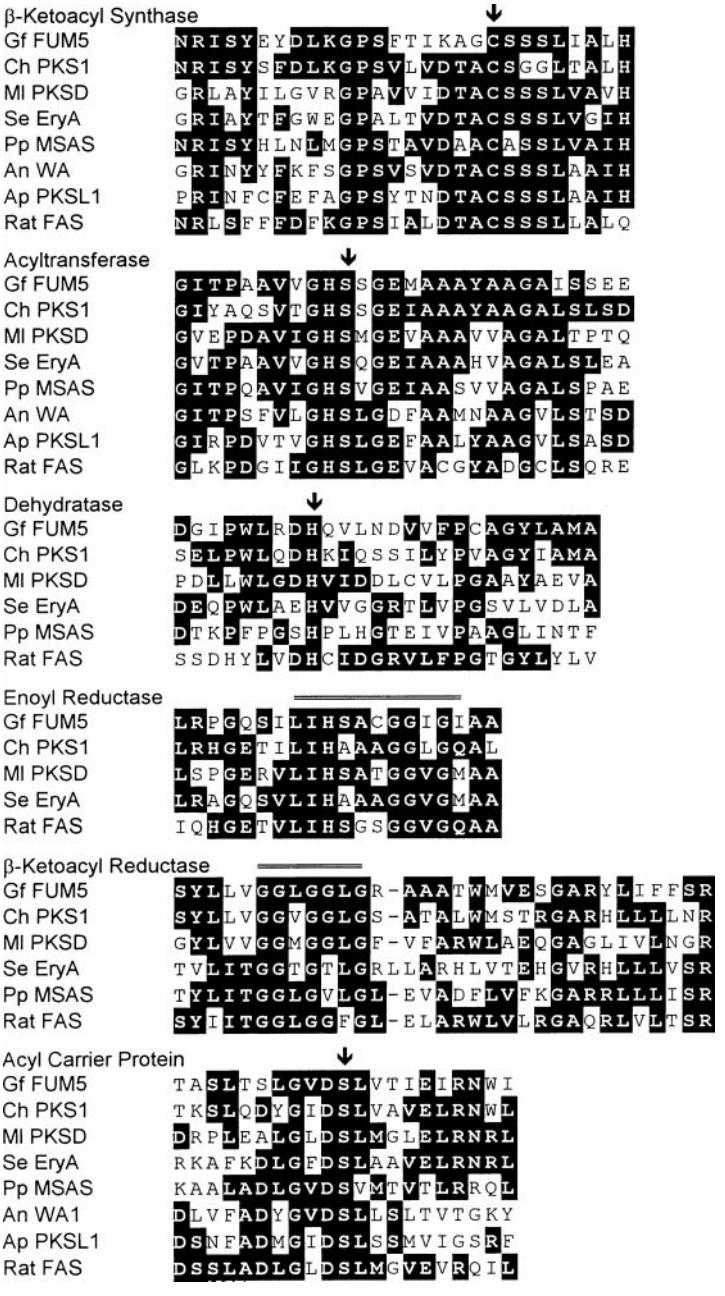

FIG. 4. Alignment of the *G. fujikuroi* MP-A *FUM5* functional domains with those from other Type I PKSs and the FAS from rat. Areas in black indicate identical amino acids. Arrows and overlines indicate active sites identified in other systems. Abbreviations: Gf FUM5, *G. fujikuroi* MP-A *FUM5* product; Ch PKS1, *C. heterostrophus PKS1* product (Yang *et al.,* 1996); M1 PKSD, *Mycobacterium leprae pksD* product (direct EMBL submission, Accession No. S73014); Se EryA, *Saccharopolyspora erythraea eryA* module 4 (Donadio *et al.,* 1991); Pp MSAS, *Penicillium patulum* MSAS (Beck *et al.,* 1990); An WA, *Aspregillus nidulans wA* product (Brown *et al.,* 1996); Ap PKS1, *A. parasiticus pksl1* product (Feng and Leonard, 1995), Rat FAS, rat fatty acid synthase (Amy *et al.,* 1989).

the 5′ end of the *FUM5* coding region and 22 kb of 5′ flanking DNA (Fig. 5). To determine whether cosmids 2-2 and 16-1 carried any genes corresponding to the *fum* loci previously identified via meiotic analysis of *G. fujikuroi* MP-A (Desjardins *et al.*, 1996, 1992), the cosmids were transformed into mutant strains with altered fumonisin production resulting from defective *fum* loci (Table 1). Ten to 12 transformants from each strain–cosmid combination were assayed for fumonisin production in liquid GYAM. When fumonisin-nonproducing strain 57-7-7 (*fum1*$^-$) was transformed with cosmid 16-1, the resulting transformants produced the wild-type profile of fumonisins: FB_1, FB_2, and FB_3 (Fig. 5). In contrast, transformation of strain 57-7-7 with cosmid 2-2 did not yield fumonisin-producing transformants (Fig. 5). Transformation of the FB_2-producing strain 109-R-20 (*fum2*$^-$) and the FB_3-producing strain 575-R-5 (*fum3*$^-$) with either cosmid 2-2 or 16-1 resulted in a 3- to 18-fold increase in fumonisin production among the transformants (Fig. 5). However, the transformants did not exhibit qualitative changes in fumonisin production. Transformants of strain 109-R-20 produced FB_2 only, while transformants of strain 575-R-5 produced FB_3 only. In addition, transformation of wild-type strain M-3120 with cosmid clone 2-2 or 16-1 resulted in 4- to 6-fold increases in fumonisin production, but did not cause qualitative changes in production (Fig. 5).

Disruption of FUM5. A *Bam*HI fragment corresponding to nucleotides 247-5007 of the *G. fujikuroi* MP-A *FUM5* coding region was cloned into pBluescriptII KS to construct the disruption vector. Nucleotides 2471-3004 of the *Bam*HI fragment were excised by *Hind*III digestion and replaced by a 2.5-kb *Hind*III fragment carrying the *Hyg* marker gene (Fig. 6A). Transformation of the resulting plasmid, pB6SC-Hyg, into the fumonisin-producing wild-type strain M-3125 yielded 74 hygromycin B resistant transformants. HPLC analysis of single-spored isolates of these transformants revealed that 16 did not produce detectable levels of fumonisins in GYAM (Fig. 7). In this medium, the wild-type progenitor strain M-3125 produced 57–103 μg FB_1/ml culture and other transformants produced 4–145 μg FB_1/ml culture. In cracked maize cultures, fumonisin production by the same 16 transformants was reduced by over 99% compared to the progenitor strain M-3125, according to HPLC and HPLC mass spectrometric analyses. Strain M-3125 produced 3894–4946 μg FB_1/g cracked maize while the 16 transformants produced 10–36 μg FB_1/g cracked maize. In addition, strain M-3125 produced 1875–2613 μg FB_2 and 646–693 μg FB_3/g

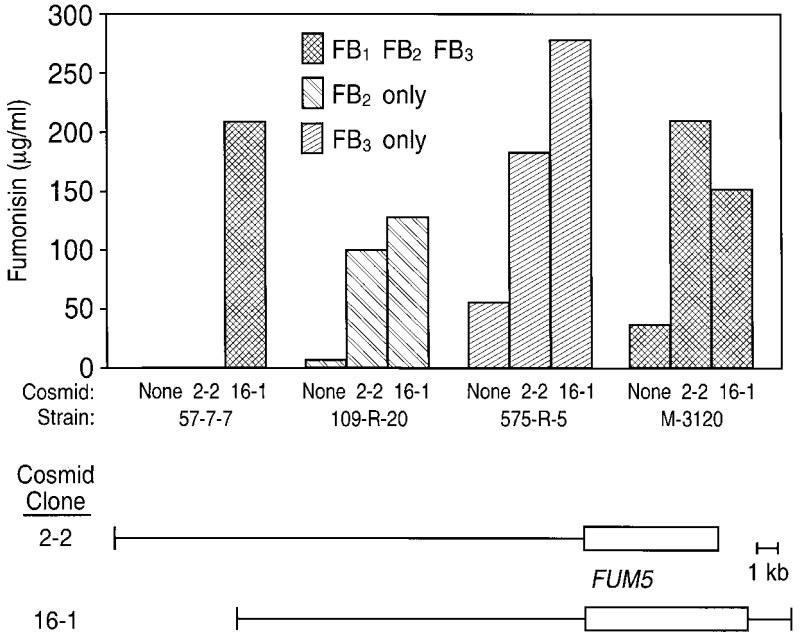

FIG. 5. (Top) Fumonisin production following transformation of *G. fujikuroi* strains 57-7-7 (nonproducer), 109-R-20 (FB_2 producer), 575-R-5 (FB_3 producer), and M-3120 (FB_1, FB_2, and FB_3 producer) with cosmids 2-2 and 16-1. Values are the means for 10 to 12 transformants grown 2 weeks in GYAM. (Bottom) Maps of cosmid clones 2-2 and 16-1 showing position of *FUM5* within each clone.

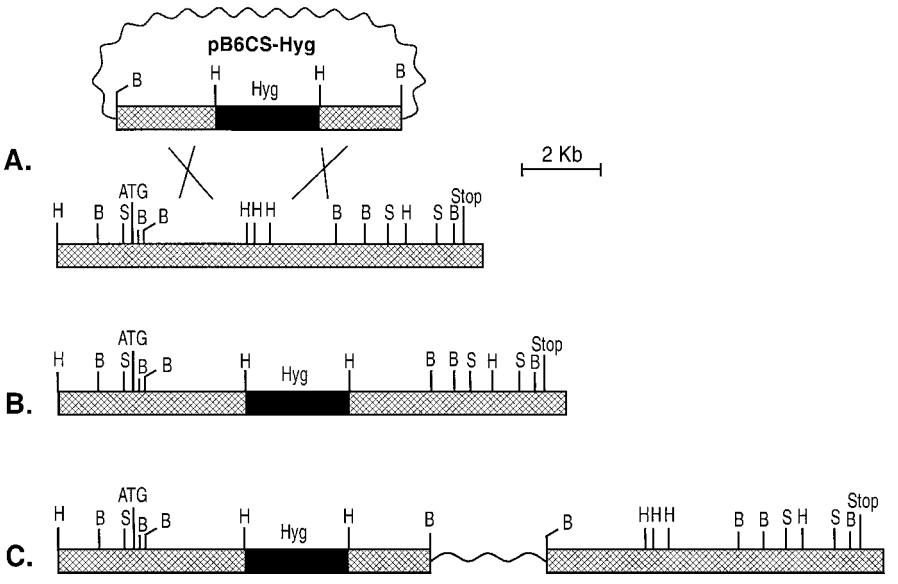

FIG. 6. Targeted gene disruption of *FUM5*. (A) Disruption vector pB6CS-Hyg and wild-type *FUM5*. (B) Gene replacement disruption of *FUM5* as occurred in transformant GFA2364. (C) Additive gene disruption as occurred in transformant GFA2186. Hyg indicates the *Hyg* marker gene, B, H, and S indicate positions of *Bam*HI, *Hind*III, and *Sma*I restriction sites, respectively, ATG and Stop indicate the positions of the start and stop codons, respectively.

cracked maize, whereas the 16 transformants did not produce detectable levels of FB_2 or FB_3. The other 58 transformants produced 1334–5183 μg FB_1, 254–3571 μg FB_2, and 85–1306 μg FB_3/g cracked maize.

Southern blot analysis revealed that the disruption vector had integrated via homologous recombination at *FUM5* in the 16 transformants with >99% reduction in fumonisin production. The hybridization pattern for only one of these transformants, GFA2364, was consistent with two homologous integration events resulting in gene replacement of *FUM5* (Fig. 6B, Fig. 8 lane 2). Hybridization patterns for 11 transformants were consistent with integration of the entire disruption vector at *FUM5* (Fig. 6C, Fig. 8 lane 3). The hybridization patterns for the remaining four transformants with low fumonisin production were consistent with the presence of more than one copy (or partial copies) of the transformation vector at *FUM5* (Fig. 8 lane 5). Southern analysis also revealed that the disruption vector integrated ectopically in transformants that exhibited wild-type fumonisin production (Fig. 8 lane 4).

DISCUSSION

The results reported here indicate that we have isolated a Type I PKS gene (*FUM5*) that participates in fumonisin biosynthesis in *G. fujikuroi* MP-A. This conclusion is based on the observations that the predicted *FUM5* translation product (Fum5p) is highly similar to fungal and bacterial Type I PKSs and that disruption of *FUM5* reduced fumonisin production by over 99% in *G. fujikuroi.* Our current hypothesis is that Fum5p catalyzes the synthesis of the fumonisin backbone from C-3 to C-20, which precursor feeding studies indicate is derived from acetate (Blackwell *et al.,* 1994). Although Fum5p also exhibits similarities to FASs we concluded that it is a PKS because it has the putative KS, AT, DH, ER, KR, and AC domains on a single translation product like other fungal PKSs. In fungal FASs these six domains and a M/PT domain are distributed between two polypeptides, FAS1 and FAS2 (Hopwood and Sherman, 1990).

The finding that *FUM5* encodes a PKS supports the hypothesis that fumonisins are produced via polyketide biosynthesis rather than via fatty acid biosynthesis. However, the putative polyketide synthesized by Fum5p is probably a completely saturated, 18-carbon molecule resembling or identical to the fatty acid stearate. This is evident from sequence data, which indicate that Fum5p has all three functional domains (KR, DH, and ER) required to fully reduce β-carbonyls to saturated carbons, and from precursor feeding studies, which indicate that the oxygen atoms attached to the fumonisin backbone are

derived from molecular oxygen rather than from β-carbonyl oxygens (Caldas *et al.*, 1998). As far as we are aware, fumonisins are the first example of a fungal polyketide synthesized by removing all oxygens from β-carbonyl carbons during formation of the polyketide chain and then later reoxygenating some of these same carbons, presumably by oxygenases after synthesis of the chain is complete. Two other molecules that may also be synthesized in this manner are the AAL toxins produced by *Alternaria alternata* and T-toxins produced by *C. heterostrophus.* Both types of toxins are linear molecules with oxygen atoms attached at various positions along a backbone. However, it is not clear whether AAL toxin is produced via a PKS (Bottini *et al.*, 1981) and although T-toxin is produced via a PKS, encoded by the *PKS1* gene (Yang *et al.*, 1996), it not known whether the oxygen atoms attached to the polyketide backbone originate from β-carbonyl groups or from molecular oxygen. If the oxygens are derived from β-carbonyls, the *PKS1* protein would have to process different β-carbonyls differently, leaving some unreduced while reducing

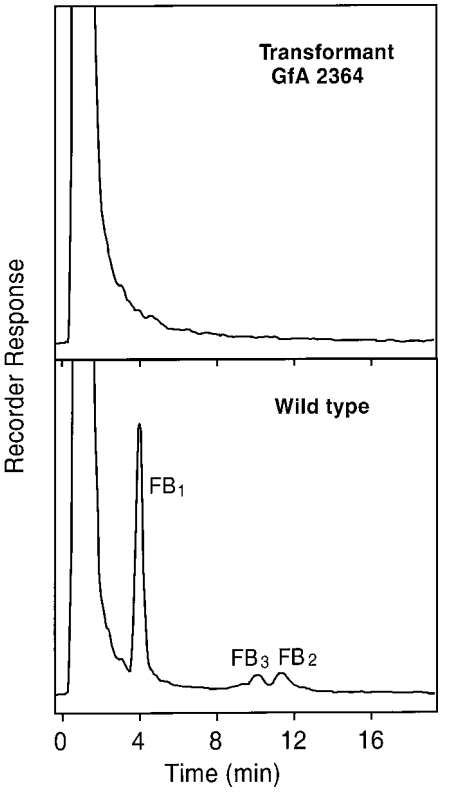

FIG. 7. HPLC Chromatograms of culture extracts from *G. fujikuroi FUM5* disruption mutant GFA2364 and wild-type strain M-3125, showing the peaks corresponding to FB_1, FB_2, and FB_3.

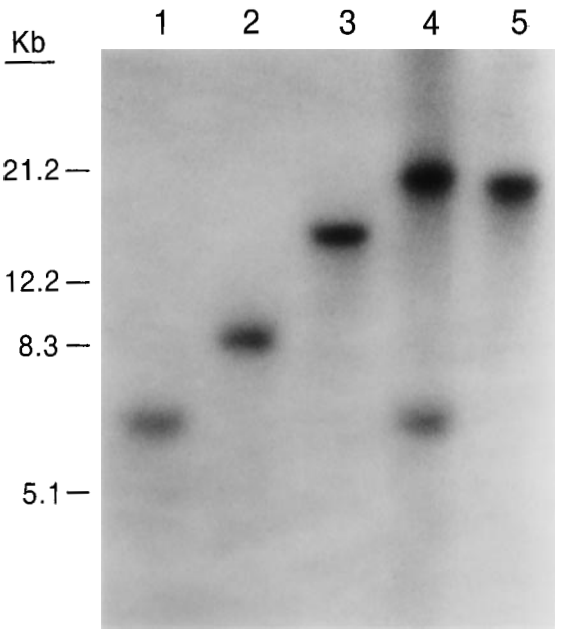

FIG. 8. Southern blot analysis of *G. fujikuroi* strains following transformation with *FUM5* disruption vector pB6CS-Hyg. Genomic DNA minipreps were digested with *Sma*I, electrophoresed on an 0.7% agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled 1.4-kb PCR-amplified fragment of the *FUM5* coding region. This fragment lay between the *Hind*III and *Bam*HI sites within the 2-kb region of *FUM5* immediately downstream of the *Hyg* insert in pB6CS-Hyg (Fig. 6). Lane 1, untransformed wild-type strain M-3125; lanes 2, 3, and 5, transformants GFA2364, GFA2186, GFA3059, respectively, with >99% reduction in FB_1 production; lane 4, transformant GFA2193 with wild-type production.

others to hydroxyls and still others to saturated carbons. Such differential processing of β-carbonyls appears to occur during the biosynthesis of the polyketide 6-methylsalicylic acid in *Penicillium patulum.* The PKS 6-methylsalicylic acid synthase leaves two β-carbonyls unreduced but reduces a third to a hydroxyl and a forth to an enoyl function prior to cyclization of the molecule (Beck *et al.*, 1990).

The complementation of the *fum1*$^-$ strain of *G. fujikuroi* by transformation with cosmid clone 16-1 indicates that a wild-type copy of the mutated DNA in this strain is present on the cosmid. Efforts are underway to define the limits of the *fum1*$^-$ locus by determining the minimal amount of clone 16-1 that complements the mutant. The fact that the *fum2*$^-$ and *fum 3*$^-$ mutants were not complemented by transformation with cosmid clone 2-2 or 16-1 suggests that wild-type copies of the DNA mutated in these two strains were not located on either cosmid. This finding is consistent with genetic linkage data that indicates *fum1* and

fum 2 are 6.2 cM apart (Desjardins *et al.,* 1996), a value that may correspond to a physical distance of 360 kb or more (Xu and Leslie, 1996).

The increased fumonisin production in the *fum2*$^-$, *fum3*$^-$, and wild-type strains of *G. fujikuroi* following transformation with cosmids 2-2 and 16-1 may have resulted from a gene dosage effect. Similar gene dosage effects have been observed in other filamentous fungi. For example, increasing the copy number of the appropriate biosynthetic genes in *P. chrysogemum* and *F. sporotrichioides* resulted in increased production of penicillin and trichothecenes, respectively (Hohn *et al.,* 1993; Barredo *et al.,* 1989). So far, the increase in fumonisin production in *G. fujikuroi* has been specific to cosmids carrying *FUM5.* Transformation with random cosmids did not result in increased production (data not shown). However, whether the effect resulted from the presence of *FUM5* or other fumonisin biosynthetic genes that may be present on the cosmids, or both, is unclear. The increased fumonisin production is most likely not caused by *FUM5* alone because cosmid clone 2-2 caused the increase in production and yet does not carry the entire *FUM5* gene. Furthermore, it is possible that other fumonisin biosynthetic genes are present on the cosmid clones because toxin biosynthetic genes tend to be clustered in filamentous fungi (Keller and Hohn, 1997), the *fum* loci are linked (Desjardins *et al.,* 1996), and partial sequence analysis revealed the presence of three additional open reading frames on cosmid 16-1 (data not shown). Experiments are underway to determine whether these open reading frames are part of other fumonisin biosynthetic genes.

The results of this study demonstrate the utility of the degenerate KS primers in the isolation of PKS genes via PCR. However, the approach may be limited by the availability of the appropriate cDNA. We used the same KS primers in PCR with genomic DNA templates from *G. fujikuroi* to amplify seven different DNA fragments, the predicted translation products of which exhibited sequence similarity to KS domains of Type I PKSs (data not shown). However, none of these amplified fragments was the KS region of *FUM5.* In contrast, when we employed a cDNA template prepared from a fumonisin producing culture of *G. fujikuroi,* the *FUM5* KS region was the major, perhaps only, product amplified. These results suggest that with the genomic DNA template, multiple loci competed for binding of the KS primers and inhibited amplification of the *FUM5* KS region. Presumably, enrichment of the *FUM5* transcript in the cDNA template reduced this competition for primer binding and allowed the *FUM5* KS region to be amplified. Thus, the PCR approach employed in this study may be useful for isolating PKS genes involved in the biosynthesis of other fungal polyketides if the appropriate culture conditions for polyketide production and RNA isolation can be determined.

It is unclear why *FUM5* disruption mutants of *G. fujikuroi* MP-A continue to produce low levels of FB_1 in cracked maize culture. A possible explanation is that a compound from some other metabolic process that is similar in structure to the fumonisin backbone could be incorporated into the fumonisin biosynthetic pathway to yield low levels of FB_1. For example, the fumonisin backbone is almost identical in structure to stearate, an 18-carbon fatty acid produced by numerous ascomycetes including various species of *Fusarium* (Weete, 1980). Fumonisin biosynthetic enzymes might be able to aminate, methylate, hydroxylate, and esterify stearic acid to yield FB_1. It is unlikely that the low level FB_1 production was due to reversion of the disrupted *FUM5* to wild type. Although *FUM5* reversion may have been possible in disruptants with duplicated regions of *FUM5* (e.g., strain GFA2186, Fig. 6C) reversion would have been extremely unlikely in disruptant GFA2364, which did not have duplicated *FUM5* sequences. It is also unlikely that a second PKS gene contributes to the low levels of FB_1 in disruption mutant cultures because Southern blot analysis indicated that there is only one copy of *FUM5* in the *G. fujikuroi* MP-A genome (data not shown).

To our knowledge, this is the first report of the isolation and characterization of a gene involved in fumonisin biosynthesis. The isolation of *FUM5* should contribute to several areas of research. The *FUM5* disruption mutants should aid in determining the role of fumonisins in the ecology of *G. fujikuroi* MP-A. For example, we plan to compare the ability of *FUM5* disrupted and wild-type strains of the fungus to cause disease on maize and to compete with other fungal species in maize ears or on plant debris. *FUM5* disruption mutants may also be useful in animal feeding studies by aiding in the identification of metabolites other than fumonisins that contribute to the toxicity of *G. fujikuroi* MP-A cultures (Leslie *et al.,* 1996). In addition, *FUM5* should be useful for isolating homologues from other fumonisin-producing species and perhaps for isolating an putative *A. alternata* PKS gene involved in the biosynthesis of AAL toxins, which are structurally very similar to fumonisins (Bottini *et al.,* 1981). Finally, because genes involved in fumonisin biosynthesis appear to be clustered (Desjardins *et al.,* 1996), character-

ization of cosmid clones carrying *FUM5* should facilitate the identification of other fumonisin biosynthetic genes.

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